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(54) Title: DEVICE AND METHOD FOR ELECTROIMMUNOASSAY

(57) Abstract

A specific binding assay is described, having a matrix which provides for incorporation of a defined volume of liquid sample, two or more electrodes, a reversibly inactivated enzyme, a first binding partner specific for binding with the analyte in the sample, and a second binding partner which competes with the analyte for binding to the first binding partner or binds to the analyte, which is labeled with an agent capable of reversing the reversible inactivation. Upon hydration with a sample, the analyte and second binding partner compete for binding with the first binding partner. Labeled binding partner which does not bind to the immobilized binding partner is able to diffuse to the enzyme, where it reactivates the enzyme and thus produces an electrical signal.

Antibodies
Polynucleotides

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# DEVICE AND METHOD FOR ELECTROIMMUNOASSAY

## Description

### Technical Field

This invention relates to the field of specific binding assays. More particularly, the invention relates to novel immunoassay- and polynucleotide hybridization-type devices and methods which provide inherent separation of bound and free species and produce a quantitative electrical output proportional to analyte concentration.

### Background of the Invention

Specific binding assays, such as immunoassays and nucleotide hybridization assays, ideally provide a clear and detectable concentration-dependent signal in the presence of the analyte sought and do not provide a signal in the absence of the analyte. Generally, immunoassays are based on the high specificity obtainable with antibodies and the great diversity of antigens that may be detected. Polynucleotide hybridization assays (e.g., dot blots) make use of DNA and/or RNA base pairing and rely on the high specificity obtainable with polynucleotide probes of sufficient length and the control over specificity of hybridization

available by choice of conditions (particularly temperature).

There are many methods for detecting antigen-antibody binding, most of which rely on a label attached to an antibody or to a competing antigen. Labels may provide signals detectable as fluorescence, radioactivity, absorbance, color, X-ray diffraction or absorption, magnetism, enzymatic activity, and the like. Suitable labels include fluorophores, chromophores, radioactive atoms (particularly $^{32}P$ and $^{125}I$), electron-dense reagents, enzymes, and ligands having specific binding partners which are themselves detectable.

The most common types of immunoassay are based upon competitive and immunometric (sandwich) methods, originally developed using radioactive labels detected by scintillation counting. More recently, analogous methods using nonisotopic labels have become more popular than radioimmunoassay (RIA) for reasons of safety, convenience, and reagent shelf-life. Currently most prevalent, enzyme immunoassay (EIA) relies upon attachment of an enzyme which catalyzes a signal-generating (usually colorimetric) reaction to an antibody or competing antigen. While EIA results may be interpreted qualitatively by visual inspection, quantitative results are generally obtained by spectrophotometric methods. Nucleotide hybridization assays can also be developed using either isotopic or nonisotopic labels. Radioactive labels, particularly $^{32}P$, are presently most common, but chemiluminescent and enzymatic labels for nucleic acid probes are gaining widespread attention.

Specific binding assays are generally categorized as either heterogeneous or homogeneous, depending on whether the bound species and free species must be separated following the binding reaction. In a homogeneous assay, the binding reaction alters the activity of the label, allowing

its distinction from non-bound label without the need for physical separation. In a heterogeneous assay, binding does not alter the label activity, thus requiring separation of bound from non-bound species prior to quantifying the signal. Conventional heterogeneous immunoassays generally rely upon solid phase separation techniques. One binding partner (antibody or antigen) is typically immobilized at a support surface (such as a microtiter dish well) in order to allow separation. Addition of a solution containing the other suitably labeled specific binding partner results in binding at the support surface. The reaction mixture is then decanted, and the solid phase is washed thoroughly to remove any non-bound species. Binding is then detected, usually by adding a solution whose composition is optimized to maximize signal-emitting activity of bound label. In EIA, a solution is added containing a substrate for the enzyme and an indicator, which is generally a chemical which changes color after the enzyme-catalyzed reaction. One commonly used EIA configuration is the enzyme-linked immunosorbent assay (ELISA), a sandwich-type assay in which antigen-dependent binding of a solution-phase "primary" antibody is detected through use of a labeled "secondary" antibody. A large number of ELISA assays can be performed simultaneously using antibody-coated 96-well microtiter plates. Wells containing antigen will bind the primary and second antibody, thus allowing the labeling enzyme to catalyze the signal-generating reaction: in the most common ELISAs, positive wells develop color, the intensity depending on antigen concentration, and hue depending on the choice of enzyme and indicator. Negative wells contain less antigen than the detection limit of the assay and fail to catalyze the reaction, thus remaining clear. A more accurate reading may be obtained by using a spectrophotometer to determine the optical absorbance at the appropriate wavelength. Heterogen-

eous assays are in general simple to perform, but accuracy and precision are contingent upon separation efficiency, which may be both operator- and sample-dependent. Variations in non-specific binding and in dissociation of bound label during washing are common sources of error in heterogeneous assays. Heterogeneous assays employing microparticulate solid phases are also subject to variable solid phase recovery with separation and washing.

Homogeneous assays do not require separation or washing steps because the binding reaction directly affects the label activity. In the EMIT assay, antibodies or antigens are labeled with an enzyme which is linked in such a manner that antigen-antibody binding blocks or masks the enzyme active site, thus preventing catalysis of the signal-generating reaction. Absent the need for separation, homogeneous assays can be performed in solution. The solution-phase reaction is generally more rapid than the reaction in heterogeneous assays, which tend to be diffusion-limited. Further, by obviating the need for washing steps, homogeneous assays avoid the introduction of imprecision inherent in heterogeneous assays. However, homogeneous assays can be exceedingly difficult and expensive to develop. This is because one cannot predict a priori whether a given labeling technique will result in a conjugate whose signal-generating activity will be influenced by the antibody binding reaction. Different labeling methods must therefore be coevaluated with different antibodies to select reagents amenable to homogeneous formats, and there is no guarantee that a suitable reagent combination will be identified. Homogeneous methods are also limited with respect to the type of label employed and the scope of analytes which can be effectively measured. Isotopic labels, for example, are not suitable for homogeneous assays, because radioactive decay is not influenced by chemical or physical parameters. Due

to the stearic constraints of the label-species interaction, the analyte must typically be a small molecule (e.g., thyroxin, theophylline, digoxin, codeine, etc.). Additionally, since these reactions are characterized by the absence of a separation step, the signal-generating reaction is subject to interferences from substances present in the sample, such as endogenous enzymes, enzyme inhibitors, substrates or cofactors, fluorescence quenchers, and the like.

Janata et al, U.S. Pat. No. 3,966,580 disclosed an electrode coated with a hydrophobic membrane, having antibodies (or antigen) immobilized on the surface. Antigen binding is detected by the resulting change in potential. This device lacks the enzymatic amplification associated with ELISA assays, and thus exhibits a relatively low sensitivity.

Boguslaski et al, U.S. Pat. No. 4,230,797, disclosed a heterogeneous immunoassay system using coenzymes to label known antigens in a competitive assay. To detect an unknown concentration of ligand, Boguslaski used a known quantity of ligand coupled to a coenzyme (e.g., NADP, coenzyme A, FAD, etc.), an immobilized specific binding partner for the ligand (e.g., an anti-ligand antibody) and an enzyme which is assisted by the coenzyme (e.g., glucose-6-phosphate dehydrogenase, D-aminoacid oxidase, etc.). After extensive washing to effect separation, the reaction is detected by the typical colorimetric, fluorometric, etc., techniques, the preferred method being chemiluminescent. One or more of the components may be provided on a carrier, which is preferably an insoluble, porous, absorbent matrix.

Hornby et al, U.S. Pat. No. 4,238,565, disclosed a method for determining the concentration of a ligand in solution, using ligand (or an analog) labeled with an enzyme prosthetic group (for example FAD), a ligand-binding molecule (e.g., an antibody) and an apoenzyme. The preferred

system used FAD as the labeling prosthetic group and apoglucose oxidase as the apoenzyme. In this case, the assay relied on the fact that an apoenzyme exhibits no activity until reconstituted with its prosthetic group. Homogeneous immunoassay methods were disclosed wherein reconstitution of the apoenzyme by prosthetic group-labeled ligand is inhibited by anti-ligand antibody. Heterogeneous methods were described wherein antibody binding did not influence the ability of the labeled ligand to reconstitute apoenzyme activity, such that physical separation of bound and free species was required. The signal in each case is provided by colorimetric methods.

Rupchock et al, U.S. Pat. No. 4,447,526, disclosed a homogeneous immunoassay system using apoglucose oxidase, a FAD-labeled ligand, and a ligand-binding partner whose binding to the labeled species inhibits apoglucose oxidase activation. In this case, the reagents are absorbed on a filter paper or other matrix, which may include several components. Detection was colorimetric.

Greenquist et al, U.S. Pat. No. 4,447,529, disclosed a homogeneous immunoassay system using FAD bound to a ligand, a ligand-binding partner and apoglucose oxidase. As in Rupchock, the reagents were absorbed on a filter paper or other matrix, and binding inhibited the ability of FAD-ligand to activate apoglucose oxidase. The patent presented examples of fluorometric and colorimetric determination of theophylline using FAD-apoGO.

McConnell, U.S. Pat. No. 4,490,216, disclosed a device comprising a conductive surface coated with a hydrophobic bilayer, where the outer layer carries hydrophilic groups and binding molecules (e.g., antibodies). Ligands binding to the device are detected by the change in capacitance. An alternative embodiment is also suggested in which the ligands are labeled with apoenzymes. Antibodies are

used which interfere with apoenzyme reconstitution when bound to the labeled ligand.

Bowers et al, U.S. Pat. No. 4,704,193, disclosed electrodes having bound cofactors (such as FAD), in order to electrically modulate enzyme-catalyzed reactions. In this case, the active form of the flavin group is regenerated electrically, rather than by redox carriers.

Forrest et al, EPO 142,301 disclosed an immunoassay employing "redox centers" as labels. Binding a redox-labeled antigen or antibody perturbs the electrochemical reaction, providing an amperometric signal.

Durfor et al, U.S. Pat. No. 4,797,181, disclosed an electrode having a cofactor (such as FAD) immobilized on its surface, providing for direct electron transport after reconstitution with an apoenzyme. The patent mentioned that the electrode could be developed into bioelectronic detectors but failed to suggest methods or details of construction.

One disadvantage of most current immunoassays is that they require multiple reagent additions and/or handling steps. Reagents often require precision pipetting with attention to order of addition, timing and degree of mixing. When supplied in liquid form or reconstituted from lyophilized form, reagents may lose activity with time, requiring frequent recalibration and quality control measures. Most labels presently in use have a limited dynamic range, such that patient samples yielding discordant results must be diluted and retested. Further, most immunoassays (particularly homogeneous assays) are sensitive to interference when testing complex solutions such as whole nonpurified bodily fluids. Nearly all specific binding assays require some form of sample pretreatment, ranging from simple dilution to sophisticated chemical extraction. Additionally, all heterogeneous assays require a separation step, and

immunometric methods require repeated washing to remove excess labeled antibody. The separation step introduces a source of error, as non-bound label may not be quantitatively removed and bound label may be accidentally lost during the wash.

The net effect of these drawbacks is that the results obtainable with any given assay may be dependent on the skill and patience of the operator. The operator must pay close attention to dilution volumes, reaction times, temperature, and washing protocols in order to obtain reliable, reproducible results. This in turn limits the market for such assays to dedicated operators and laboratories, for example commercial testing services, hospitals and medical clinic labs.

## Disclosure of the Invention

We have now invented a new specific binding assay, which is capable of analyzing complex fluids such as whole blood without operator pretreatment, which is not sample volume or interference sensitive, and which provides a quantitative electrical signal, thus obviating reliance on chromogenic reagents, spectrophotometers and the like. The assay requires only an approximate volume of an untreated sample and provides a quantitative reading without further manipulation by the operator. The assay of the invention combines the versatility of heterogeneous assay with the speed, accuracy and convenience of homogeneous assay.

The assay device of the invention has a surface comprising a working electrode, and a reference electrode. Immobilized at the working electrode is one member of a reversibly inactivated enzyme/reactivating agent set, where the enzyme (when activated) is capable of catalyzing a current-generating reaction. The reactivating agent spontaneously reconstitutes the enzyme on contact, and restores

activity. Covering this first set member and the working electrode is a swellable matrix, which separates the two set members and prevents their reconstitution in the absence of a liquid sample. Within or above the matrix is a first binding partner which is specific for the analyte, and a second binding partner which competes with the analyte for binding to the first binding partner, where either the first or the second specific binding partner is labeled with the other member of the reversibly inactivated enzyme/reactivating agent set. Alternatively, the second binding partner may bind to the analyte, as in a sandwich-type assay. The matrix swells on contact with the analyte sample, drawing a predetermined volume of the sample in rapidly. Optionally, the matrix may effect an initial filtration of the sample, for example by excluding whole cells, fibrous proteins, and the like. Upon hydration by the sample, the analyte and second binding partner compete for binding with the first binding partner. The labeled binding partner which does not bind to the other binding partner is able to diffuse to the working electrode, where the label and reversibly inactivated enzyme reconstitute the enzyme activity and thus produce a quantifiable electrical signal.

Another aspect of the invention is a multiple analyte detection device, which comprises a plurality of detection modules, each modules consisting of an assay device as described above. The individual modules may differ from each other by having different specificities for their specific binding partners, by having binding partners specific for the same analyte but having different binding affinities, by having affinities for different epitopes of the same analyte, by employing different mediators or enzyme systems, and the like. The device may further include sample receiving means, for distributing portions of the liquid sample to each of the detection modules.

Another aspect of the invention is a method for assaying a liquid sample for an analyte, which method comprises applying a liquid sample to one of the above-described devices.

Brief Description of the Drawings

Figure 1 illustrates a representative device of the invention, having surface 1, working electrode 2, reference electrode 3, reversibly inactivated enzyme 4 immobilized at working electrode 3, matrix 6 containing immobilized binding partner A (5), labeled binding partner B (7) labeled with reactivating agent L (8), and signal detecting means 9.

Figure 2 graphically depicts current dependence on glucose oxidase concentration ($\mu$A/min vs. M), as described in Example 1, using a carbon disk electrode at 600 mV vs. AgCl in 6 mL of solution containing 5 mM benzoquinone, 5 mM glucose, 5 mM $NaN_3$.

Figure 3 depicts increase in current with FAD concentration ($\mu$A vs. M), as described in Example 2A, using a sputtered Pt electrode vs. sputtered Ag at 450 mV (vs. AgCl) in a 13 mL solution of 50 $\mu$g/mL apoGO, 50 mM benzoquinone, 50 mM glucose, and 50 mM $NaN_3$.

Figure 4 shows the percent increase in current with FAD concentration using apoGO immobilized on a sputtered Pt electrode in a polyacrylamide gel, in 8 mL of 15 mM benzoquinone, 37.5 mM glucose at 600 mV vs. AgCl, as described in Example 2B.

Figure 5 shows the percent increase in oxidation current and percent decrease in reduction current with increasing FAD concentration as described in Example 3A, using a carbon disk electrode at 500 mV vs. AgCl wire (270 mV vs. AgCl for reduction current measurements) in 6 mL of

33 μg/mL apoGO, 500 mM benzoquinone, 5 mM glucose, 5 mM $NaN_3$, determined by cyclic voltammetry.

Figure 6 is a plan view of a device of the invention for assaying multiple analytes. The device comprises two or more detection modules 13, 13′, each having surface 1 and 1′, working electrode 2 and 2′ depicted here as a disk, reference electrode 3 and 3′ depicted here as a straight wire, reversibly inactivated enzyme 4 and 4′ immobilized at working electrode 3 and 3′, matrix 6 and 6′ containing immobilized binding partner A (5 and 5′), labeled binding partner B (7 and 7′) labeled with reactivating agent L (8 and 8′), and signal detecting means 9 and 9′. The device optionally further comprises signal comparing and displaying means 10, for comparing the output from two or more modules and conveying the results to the operator. The device may also include a second swellable matrix 11 which may be common to two or more modules, or may be divided into portions corresponding to individual modules. The second matrix 11 may additionally be provided with depression 12 for receiving samples: matrix 11 together with depression 12 form a sample receiving means.

Figure 7 is a top view of another embodiment of the multiple assay device of the invention. This device has sample receiving means comprising a well 21 positioned centrally on the top surface of the device, and connected to each of the individual modules 13 by channels 22 recessed into the surface of the device. When a drop of sample is placed in well 21, capillary action draws the sample into channels 22 and distributes the sample to each of the modules 13.

## Modes of Carrying Out The Invention

### A. Definitions

The term "enzyme" as used herein refers to an enzyme which is capable of catalyzing a reaction which provides an electrical signal. Suitable enzymes within the scope of this invention include, without limitation, glucose oxidase, glutathione reductase, cytochrome reductase, NADPH: oxidoreductase, lipoamide dehydrogenase, pyridoxine phosphate oxidase, horseradish peroxidase, cytochrome C, urease, alkaline phosphatase, ß-galactosidase, ß-lactamase, and the like.

The term "reversibly inactivated" refers to a state in which the enzyme is incapable of catalyzing the signal-generating reaction at significant levels. Reversible inactivation may be effected by removing the prosthetic group or coenzyme from an enzyme which requires such, or by excluding the enzyme's substrate or by exposing the enzyme to a reversible inhibitor. Many enzymes depend on prosthetic groups or coenzymes for their activity, thus rendering the corresponding apoenzymes suitable for use as reversibly inactivated enzymes. In any event, the reversibly inactivated enzyme must be capable of reactivation spontaneously upon contact with the label/reactivating agent used in the assay.

The term "reactivating agent" refers to a molecule capable of restoring enzyme activity to the reversibly inactivated enzyme. The reactivating agent may also be referred to as a "label" for the purposes of this invention. Where the reversibly inactivated enzyme is an apoenzyme, the reactivating agent will generally be a prosthetic group such as flavin adenine dinucleotide (FAD), flavin mononucleotide (FMN), heme, and the like. The reactivating agent may be an enzyme substrate, if desired, although it will be apparent

that no amplification of signal is obtained thereby. In this case, care need also be taken that the substrate is not naturally present in significant concentrations in the sample to be analyzed. The presently preferred reactivating agent is FAD, used with apoglucose oxidase.

The term "reconstituting" as used herein refers to the restoration of enzymatic activity to the reversibly inactivated enzyme by the reactivating agent. In a preferred embodiment of the invention, "reconstitution" refers to regeneration of a holoenzyme by recombination of an apoenzyme with its appropriate prosthetic group, cofactor or coenzyme. However, reconstitution also includes the provision of a substrate (for example, ATP, GTP, etc.) to an enzyme which is inactive in the absence of the substrate, and the provision of an "anti-inhibitor" to an enzyme which is reversibly inhibited.

The term "specific binding partner" refers to a molecule which binds selectively and specifically. A specific binding partner is "specific" for the molecule to which it binds, exhibiting a strong affinity for the binding partner without binding unrelated molecules. For example, the first specific binding partner may be an antibody specific for the analyte to be detected. The second specific binding partner may be a labeled analyte molecule or an analog of the analyte which is capable of binding to the other specific binding partner. Specific binding partners will generally be derived from antibodies or fragments thereof (e.g., Fab, $F(ab')_2$, etc.) and antigens corresponding to the analyte or analogs thereof, or complementary nucleic acid strands (for example, viral DNA or RNA and a complementary DNA probe), but other binding pairs such as avidin and biotin, or receptor-ligand pairs are also suitable.

The term "matrix" as used herein refers to a solid or semisolid substance which is permeable to the analyte. The matrix must be insoluble under conditions of the assay (e.g., in the presence of water, biological fluids, cellular digests, enzyme substrates and products, etc.) and is preferably hygroscopic. The matrix functions to keep the reversibly inactivated enzyme separate from the reactivating agent until the time of assay and prevents diffusion of the non-labeled specific binding partner to the enzyme. One of the specific binding partners is retained within or above the matrix, which thus effects an automatic separation. This retention may be effected either by binding the specific binding partner within the matrix or to the surface thereof, or by selecting a matrix which is impermeable to the selected binding partner. However, the membrane must be permeable to the labeled species. When the second binding partner (analyte analog or sandwich antibody) is labeled, the matrix need only be permeable to the second binding partner. When the first specific binding partner is labeled, the labeled species will generally be a complex of binding partner and analyte. In such cases, the matrix must be permeable to the complex. Suitable matrices may be formed from polyacrylamide, agarose, collagen, gelatin, starch, cellulose, polyurethane, nylon, and the like, and may take the form of a gel layer, thin membrane, bulk solid, and the like.

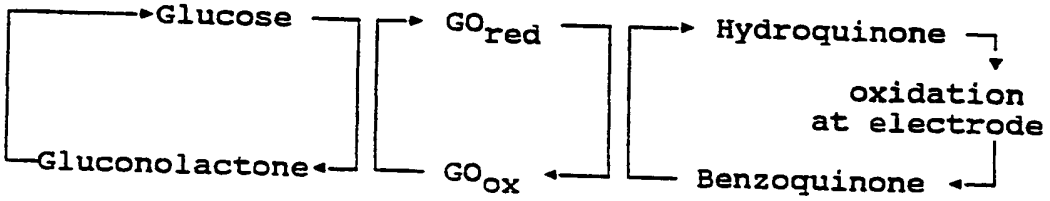
The term "signal-detecting means" as used herein refers generally to circuitry designed for discrimination between a signal and any background noise. The particular detection means employed will depend upon the form of the signal employed. For example, an amperometric device is used to detect generation of a current. An ohmmeter may be used to quantify resistance (conductivity). A voltmeter may be employed for potentiometric determinations. Other

instruments for detecting the electrochemical results of the enzyme-catalyzed reaction will be apparent to those of ordinary skill in the art and are considered equivalents herein.

The term "mediator" as used herein refers to a compound added to reduce overvoltage at the electrodes. A mediator is selected which is highly diffusible and which has a reduction potential lower than the enzyme reaction product. Exemplary mediators include, without limitation, ferrocene, hexacyanoferrate, ferricinium salts, benzoquinone, methylene blue, methyl viologen, benzyl viologen, polyviologen, and the like. Suitable mediators are selected on the basis of suitable redox potentials, absence from typical compositions to be assayed, chemical stability, lack of photosensitivity, reproducibility of results obtained and ability to react with the enzyme and the electrode surface.

The term "current-generating chemical reaction" refers to a reaction which liberates or consumes electrons, i.e., an oxidation-reduction or "redox" reaction. Any chemical reaction which produces a measurable electrical potential (voltage) or current may be employed. Suitable current-generating chemical reactions are those in which the reactants may be cycled or regenerated by reaction at an electrode surface, with or without the intervention of a mediator. In other words, any enzyme-catalyzed redox chemical reaction will be suitable if the reactants may be regenerated at an electrode surface, or if the reactants may be regenerated by reaction with a mediator which is itself regenerated at an electrode surface. An exemplary chemical reaction is the oxidation of glucose to gluconolactone by glucose oxidase. The glucose oxidase is changed from its oxidized form to its reduced form in the process. In the absence of suitable mediators, glucose oxidase produces hydrogen peroxide in the process. However, in the presence

of benzoquinone, the glucose oxidase is regenerated to its oxidized form, while the benzoquinone is reduced to hydroquinone. The hydroquinone diffuses rapidly to the electrode surface, and in the presence of a suitable voltage bias is regenerated back to benzoquinone.




Thus, reactions which are not directly accessible at the electrode surface may be used by employing a mediator capable of reacting with both the enzyme and the electrode. Other suitable reactions include, without limitation: oxidation of D-aminoacids to α-ketoacids by D-aminoacid oxidase; reduction of glutathione by glutathione reductase; reduction of benzoquinone to hydroquinone by quinone reductase; and the like. See for example, Boguslaski et al, U.S. Pat. No. 4,230,797.

Any reference to a member "immobilized at" a surface indicates that the member (either a reversibly inactivated enzyme or its reactivating agent) is positioned on or near the surface and that it is constrained from diffusion away from the surface. Immobilization includes, without limitation, chemically attaching the member to the surface (for example, crosslinking by radiation and/or addition of dialdehydes such as glutaraldehyde), physically adsorbing the member on the surface, drying the member on the surface, placing the member on the surface and covering it with a substance impermeable to the member (for example, a polyacrylamide gel, nylon membrane, etc.), entrapping the member in a matrix applied to the surface (for example, by

forming a polyacrylamide or agarose gel in the presence of the member), binding the member within a matrix (e.g., by crosslinking the member within a gel or membrane or adsorbing the member onto a membrane applied to the surface), and the like.

The term "sample receiving means" refers to an optional feature of a multiple analysis device which receives and distributes the sample to each of the detection modules (each detection module essentially constituting an otherwise independent assay device of the invention). For example, a multiple analysis device of the invention may be provided in the form of a plate having a plurality of well-like assay devices. The sample receiving means may take the form of a centrally positioned well having radiating channels or capillaries leading to each of the independent assay wells. Alternatively, the sample receiving means may comprise an analyte-permeable gel overlaying the entire device, thus insuring that sample is provided to each of the modules by diffusion.

B. General Method

1. Preparation:

The devices of the invention are based on a combination of a separatory matrix with an electrochemical signal generation and detection means, optionally further including a sample pretreatment matrix. The detection means generally comprises a working electrode and a reference electrode, optionally a third counterelectrode, coupled to circuitry for measuring the parameter of interest. The detection circuitry is selected in concert with the choice of signal-generating enzyme, as the reaction catalyzed by the enzyme will determine the most suitable signal for measurement. For example, in a presently preferred embodiment, the enzyme employed is an oxidoreductase which catalyzes the

oxidation or reduction of its substrate. This reaction is reversed at the electrode surface, providing a current which may be detected by an ammeter of suitable sensitivity. In a presently preferred embodiment of the invention, amperometric detection at fixed potential is used to measure the signal current.

The electrode used will generally be standard platinum, silver, gold, or carbon suitable for electrochemistry. The electrode surface may be modified as is common practice in electrochemistry, for example by doping carbon electrodes with anionic or cationic carriers, by chloridizing or etching metal electrodes, and the like. The electrode may take the form of a rod, disk, wire, cylinder, wire mesh, and the like. The precise form and composition of the electrode may be optimized by routine experimentation, following the examples set forth below.

The reversibly inactivated enzyme/reactivating agent set is selected based on a number of criteria. The reactivating agent may be an essential cofactor, prosthetic group or enzyme substrate. However, using the substrate as the reactivating agent does not provide amplification of the signal: reconstitution of the enzyme then provides only a 1:1 correspondence between the number of analyte molecules and the number of signal molecules produced. Accordingly, it is presently preferred to employ a cofactor or prosthetic group as the reactivating agent. In this case, restoration of enzyme activity in the presence of an excess of substrate results in amplification of the signal: a large number of signal (processed substrate) molecules is produced for each molecule of enzyme reconstituted. The amplification factor depends upon the fraction of enzyme reconstituted to full activity and the reaction rate of the reconstituted enzyme. Accordingly, enzymes having high specific activity and high reaction rates are used for applications requiring a large

degree of amplification, providing maximal sensitivity. The specific activity and reaction rate constants will generally be known for any selected reversibly inactivated enzyme/reactivating agent set or may be determined by standard biochemical methods.

Also, the reaction catalyzed by the reconstituted enzyme must provide an electrochemical signal, in the form of a current, potential or conductance. For example, where the enzyme is an oxidoreductase, a product of the reaction is generated in an oxidized (or reduced) form, which then diffuses to the working electrode, where the reaction is reversed and the reactant regenerated. For example, glucose oxidase oxidizes glucose to gluconolactone, in the process generating hydrogen peroxide. The peroxide may then be converted to water at the electrode.

Preferably, the reaction employs a mediator, for example benzoquinone. When added to a solution of glucose oxidase and glucose, benzoquinone is reduced to hydroquinone (replacing peroxide formation) which may be reoxidized to benzoquinone at the working electrode. The mediator is selected to have an oxidation (or reduction) potential lower than that of the oxidized (or reduced) reaction product. This results in a reduction of the overvoltage required at the working electrode in order to complete the reaction. Reducing the overvoltage is advantageous because it limits the number of interfering species potentially present in the assay sample which may interfere by reacting at the electrode.

Another consideration is that the reversibly inactivated enzyme/reactivating agent set must be capable of spontaneous reconstitution upon contact under the conditions present within the device of the invention. In the case of an apoenzyme/prosthetic group set, the prosthetic group must be able to reconstitute the holoenzyme when one

member of the set is immobilized near the electrode surface and the other member of the set is conjugated to a binding partner. The suitability of any selected reversibly inactivated enzyme/reactivating agent set may be determined by routine experimentation, following the examples set forth below.

There are a number of different assay configurations available for the practice of the invention. The device may be designed having a reversibly inactivated enzyme immobilized at or near the electrode surface, with an analyte-specific binding partner bound (e.g., an antibody) to a solid phase (e.g., gel layer) and free analyte (or an analog thereof) labeled with the reactivating agent (e.g., an apoenzyme prosthetic group). Alternatively, the analyte or analog may be bound to the solid (gel) phase, and the analyte-specific binding partner labeled with the reactivating agent. In the first embodiment, analyte in the sample competes with labeled analyte for binding to the solid phase binding partner. Increasing amount of analyte in the sample allow more of the labeled analyte/analog to diffuse to the inactivated enzyme, and thus provide an increased signal. In the second embodiment, analyte in the sample competes with solid phase analyte/analog for binding to the free labeled binding partner. Increasing amounts of analyte in the sample allow more labeled binding partner to reach the electrode surface, and again provide an increased signal.

The device may also be configured in a sandwich format, having a solid phase analyte-specific binding partner and a free labeled analyte-specific binding partner. In this embodiment, the analyte binds to both binding partners, and thus immobilizes the labeled species. Thus, increasing the concentration of analyte in the sample decreases the signal level.

One may also use displacement methods, in which the second binding partner (e.g., analyte/analog) and analyte-specific binding partner (one of which is bound to the solid phase) are provided in immunocomplex form. In these assays, the second binding partner is displaced by the sample analyte, and the labeled species allowed to diffuse to the reversibly inactivated enzyme.

The reversibly inactivated enzyme may be immobilized at the electrode surface by a variety of methods, including without limitation trapping in gel, covalent bonding to a suitably derivatized surface, drying on the surface and physical adsorption. See, for example, Nakamura et al, U.S. Pat. No. 4,321,123; Schall Jr. et al, U.S. Pat. No. 4,357,142; Williams et al, U.S. Pat. No. 4,414,080; and Gorton et al, U.S. Pat. No. 4,490,464, all incorporated herein by reference. Physical adsorption is presently preferred.

Alternatively, reversibly inactivated enzyme may be used to label the first or second specific binding partner by conjugation techniques known in the art of enzyme immunoassay. In this case, the reactivating agent would be immobilized or localized at or near the electrode surface by covalent attachment to the electrode or to a carrier or spacer which is bound to or entrapped near the electrode. See for example, Durfor et al, U.S. Pat. No. 4,797,181, and Bowers et al, U.S. Pat. No. 4,704,193, incorporated herein by reference in full.

Preparation of the specific binding partners will depend upon the particular form of binding partner pair selected. Antibodies specific for an analyte are prepared by conventional means. In general, the analyte is first used to immunize a suitable animal, preferably a mouse, rat, rabbit or goat. Rabbits and goats are preferred for the preparation of polyclonal sera due to the volume of serum

obtainable and the availability of labeled anti-rabbit and anti-goat antibodies. If the analyte is a small molecule (hapten), it is generally first conjugated to a large carrier molecule, such as keyhole limpet hemocyanin (KLH) or bovine serum albumin (BSA) prior to immunization. Immunization is generally performed by mixing or emulsifying the protein in saline, preferably in an adjuvant such as Freund's complete adjuvant, and injecting the mixture or emulsion parenterally (generally subcutaneously or intramuscularly). A dose of 50-200 μg/injection is typically sufficient. Immunization is generally boosted 2-6 weeks later with one or more injections of the protein in saline, preferably using Freund's incomplete adjuvant. One may alternatively generate antibodies by *in vitro* immunization using methods known in the art, which for the purposes of this invention is considered equivalent to *in vivo* immunization.

Polyclonal antisera is obtained by bleeding the immunized animal into a glass or plastic container, incubating the blood at 25°C for one hour, followed by incubating at 4°C for 2-18 hours. The serum is recovered by centrifugation (e.g., 1,000 x G for 10 minutes). About 20-50 mL per bleed may be obtained from rabbits.

Monoclonal antibodies are prepared following the method of Kohler and Milstein, Nature (1975) 256:495-96, or a modification thereof. Typically, a mouse or rat is immunized as described above. However, rather than bleeding the animal to extract serum, the spleen (and optionally several large lymph nodes) is removed and dissociated into single cells. If desired, the spleen cells may be screened (after removal of nonspecifically adherent cells) by applying a cell suspension to a plate or well coated with the antigen. B-cells expressing membrane-bound immunoglobulin specific for the antigen bind to the plate and are not rinsed away

with the rest of the suspension. Resulting B-cells, or all dissociated spleen cells, are then induced to fuse with myeloma cells to form hybridomas and are cultured in a selective medium (e.g., hypoxanthine, aminopterin, thymidine medium, "HAT"). The resulting hybridomas are plated by limiting dilution and are assayed for the production of antibodies which bind specifically to the immunizing antigen (and which do not bind to unrelated antigens). The selected monoclonal antibody-secreting hybridomas are then cultured either *in vitro* (e.g., in tissue culture bottles or hollow fiber reactors and the like), or *in vivo* (as ascites in mice).

Antibody derivatives are generally prepared by proteolytic cleavage of antibodies (usually IgG or IgM) to provide Fab or $F(ab')_2$ fragments. Digestion with pepsin cleaves the antibody molecule below the heavy chain-heavy chain disulfide bond, resulting in two (antigen-binding) Fab fragments and an Fc fragment. Digestion with papain cleaves the antibody molecule above the heavy chain-heavy chain disulfide bond, resulting in one (antigen-binding) $F(ab')_2$ fragment and an Fc' fragment. Antibody derivatives also include hybrid antibodies (an antibody where each half is derived from a different antibody, thus providing two non-identical antigen-binding sites) and chimeric antibodies (in which the antigen-binding regions from one antibody are joined to a different constant portion, typically using recombinant DNA methods). Hybrid and chimeric antibodies and their construction are described in Cabilly et al, U.S. Pat. No. 4,816,567, incorporated herein by reference.

Nucleic acid binding partners are prepared by means known to those of ordinary skill in the art, for example by cloning and restriction of appropriate sequences or preferably by direct chemical synthesis. For example, one may employ the phosphotriester method described by S.A.

Narang et al, Meth Enzymol, (1979) 68:90, and U.S. Pat. No. 4,356,270, incorporated herein by reference. Alternatively, one may use the phosphodiester method disclosed in E.L. Brown et al, Meth Enzymol, (1979) 68:109, incorporated herein by reference. Other methods include the diethylphosphoramidite method disclosed in Beaucage et al, Tetrahedron Lett, (1981) 22:1859-62, and the solid support method disclosed in U.S. Pat. No. 4,458,066. One of the binding partners will be labeled with a member of the reversibly inactivated enzyme/reactivating agent pair using conventional chemical methods. Polynucleotide assays of the invention will be conducted under temperature and solvent conditions appropriate to nucleic acid hybridization assays: the precise conditions will depend upon the length and homology of the binding partners, but are easily determined by those of ordinary skill in the art with no more than routine experimentation. For example, the hybridization temperature is typically calculated following the formula (assuming 0.9 M NaCl):

$$T(^{\circ}C) = 4(N_G + N_C) + 2(N_A + N_T) - 5^{\circ}C,$$

where $N_G$, $N_C$, $N_A$, and $N_T$ are the numbers of G, C, A, and T bases in the probe (J. Meinkoth et al, Anal Biochem (1984) 138:267-84). The device of the invention may optionally include temperature regulating means for maintaining the matrix at the optimum temperature for hybridization.

Binding partner/label conjugates (in which the label is a member of the reversibly inactivated enzyme/reactivating agent set) are also prepared by standard methods known in the art. Where the binding partner is an antibody, antibody derivative, or protein antigen and the label comprises an apoenzyme, one may employ standard procedures like those used to label antibodies with horseradish

peroxidase (HRP). Where the label is a prosthetic group, cofactor or substrate, and/or where the binding partner is other than a protein, suitable methods will generally include covalent bonds using a bifunctional spacer. See for example Boguslaski et al, U.S. Pat. No. 4,230,797.

The matrices used in the invention are prepared by conventional means or are obtained from commercial sources. Suitable matrices may be formed from polyacrylamide, agarose, collagen, gelatin, starch, cellulose, polyurethane, nylon, and the like, and may take the form of a gel layer, thin membrane, bulk solid, and the like. The matrix may be formed *in situ* on the electrode surface or may be preformed and cut to fit the device. The matrix material may be selected for adhesion to the electrode surface or may be held in place by adhesives or mechanical means (for example clips, retaining walls, etc.). The particular material selected will depend in part upon the configuration of the assay and the size and nature of the labeled and non-labeled binding partners. For example, where the labeled binding partner is a small organic molecule (e.g., a drug analog), the matrix need only be permeable to the labeled molecule, and need not be permeable to proteins. However, where the labeled binding partner is an antibody, the matrix must be permeable to the antibody, and generally to the antibody/analyte complex (particularly where the analyte is a large protein). In such cases, the matrix will generally be a gel such as polyacrylamide, the permeability of which can be varied by controlling the degree of polymerization.

The supporting surface of the device must be chemically stable and generally inert under the assay conditions. Typical surface materials are nylon, polyurethane, polystyrene, polypropylene, glass, stainless steel, and the like. The supporting surface may consist almost entirely of the working electrode and/or the reference electrode, in

which case suitable materials further include platinum, silver, gold, carbon, and other typical electrode materials. The device may be structured in a variety of shapes, for example as a well, open tube, dipstick or probe, grid, or a simple flat surface. The reference electrode need not be physically attached to the remainder of the device, as long as it is electrically connected and placed in sufficient proximity to the working electrode to serve as a reference electrode. Thus, for example, one can prepare a device of the invention for assaying body fluids *in situ*, using a small probe-shaped working electrode/matrix and a separate reference electrode. This embodiment can be used to assay, for example, amniotic fluid, cerebrospinal fluid, blood components, and the like, either by insertion through the skin or by endoscopic positioning. In such applications, it is desirable to protect the matrix from contact with fluid until positioned, for example by sheathing or capping the matrix in such a manner that it may be exposed remotely.

Devices of the invention for the assay of multiple analytes may be similarly prepared. It is convenient to provide a series of detection modules on one surface, each module constituting a complete assay device. The multiple analyte device of the invention may conveniently be provided with a single sample receiving means, such that one sample placed on the device at the appropriate location will be spread and distributed to each of the modules for assay. Thus, for example, one may assay a drop of blood for a series of possible constituents simultaneously.

The devices of the invention may be assembled by a variety of methods. In a first type of device, the reversibly inactivated enzyme/reactivating agent set comprises an apoenzyme oxidoreductase and its prosthetic group (for example, apoGO and FAD). The apoenzyme is immobilized at the surface of an electrode (preferably a carbon disk or plat-

inum wire) by drying an apoGO solution on the electrode surface.

C. Examples

The examples presented below are provided as a further guide to the practitioner of ordinary skill in the art and are not to be construed as limiting the invention in any way.

Example 1

(Selection of a Mediator)

A redox couple may be evaluated for suitability as a mediator in the device of the invention using the following experiment. In this example, the benzoquinone/hydroquinone redox couple was evaluated.

(A) Redox Potentials:

A three electrode system was employed, using a working electrode of platinum (or gold) sputtered over a thin alumina plate, a counter electrode of silver sputtered over a thin alumina plate, and a reference electrode of chloridized silver wire or Ag/AgCl in saturated KCl. The electrodes were cleaned by rinsing with ethanol followed by distilled water. Phosphate buffer (0.1 M $K_2HPO_4$, 0.1 M $KH_2PO_4$, 0.1 M KCl), pH 7.4, was used to prepare all solutions. Cyclic voltammograms (-0.7 V to +0.8 V vs. AgCl, 100 mV/s scan rate) of both 10 mM benzoquinone and 10 mM hydroquinone solutions were measured with a BAS CV-37 potentiostat and recorded with an HP-7004B X-Y recorder. All measurements were taken at room temperature.

Cyclic voltammograms of the phosphate buffer alone showed no redox activity. Cyclic voltammograms of benzoquinone showed reversible redox behavior with a 6 μA reduction peak at 250 mV vs. AgCl, indicating reduction of benzoquinone to its reduced form, hydroquinone, and a 1 μA oxidation peak at 600 mV vs. AgCl, indicating oxidation of the

reduced form back to benzoquinone. The dominant reduction peak indicates that benzoquinone initially exists in excess over hydroquinone, which must be generated electrochemically.

Cyclic voltammograms of hydroquinone showed reversible redox behavior with a 5 μA oxidation peak at 600 mV vs. AgCl, indicating oxidation to benzoquinone, and a 1 μA reduction peak at 250 mV vs. AgCl, indicating reduction of the oxidized form back to hydroquinone. The dominant oxidation peak is due to the initial excess of hydroquinone over benzoquinone.

Since the present invention depends on measurement of the extent of enzyme reaction by measuring consumption of the oxidant (benzoquinone), benzoquinone was selected as the oxidant reagent for use in the examples below. However, it should be noted that benzoquinone is photosensitive. In the examples below, the concentration of benzoquinone was reduced from 10 mM. Alternatively, one may protect the reagent from light using standard masking techniques or select a photostable mediator.

(B) Oxidation of Glucose Oxidase:

Benzoquinone was next examined for ability to oxidize the reduced form of glucose oxidase (GO) in solution, by amperometric measurement at a constant potential bias.

Amperometric measurements were made with a BAS CV-27 potentiostat and recorded with a SOLTEC chart X-Y recorder. A two electrode system was employed, using a carbon disk electrode (BAS, 2 mm diameter) as the working electrode and a chloridized silver wire as the reference/counter electrode. All measurements were made in a 15 mL reaction vessel, with the electrode system immersed in 6 mL of a reaction mixture of benzoquinone, glucose and $NaN_3$ (5 mM each). The reaction mixture was stirred constantly using a 1 cm stirring bar and a NUOVA magnetic stirrer set at a stir

speed of 2. Hydroquinone oxidation current was measured at a constant potential of 600 mV vs. AgCl.

Reagent grade glucose oxidase (*Aspergillus niger*, 255 U/mg) was obtained from Sigma Chemical Co. After the baseline current was established, GO was added in 20 μL aliquots, and the change in current measured. The results are depicted graphically in Figure 2. Oxidation current increased with increasing concentration of GO, indicating that benzoquinone was able to reoxidize reduced GO in bulk solution. The useful dynamic range of the system was found to be approximately 0.01 to 20 μg/mL GO.

## Example 2

(Reconstitution of Apoenzyme)

(A) Reconstitution in Solution:

The ability of flavin adenine dinucleotide (FAD) to complex with and reactivate apoGO in a bulk solution containing all the reagents was investigated by amperometric detection at a constant potential bias.

The same sputtered Pt and Ag electrode system described above was adopted, except that only a single Ag electrode was used in a two-electrode system. All other equipment and settings were as described above. Apoglucose oxidase (apoGO) was prepared from the same lot as the active GO used in Example 1 above. The prosthetic group, FAD, was dissociated from the apoenzyme using methods described in the literature (D.L. Morris et al, Meth Enzymol (1983) 92: 413-42, incorporated herein by reference). The reactivated apoenzyme exhibited a reduced specific activity of 43 U/mg relative to the native GO starting material.

All measurements were made in a 15 mL reaction vessel containing 6.5 mL of 100 mM glucose/$NaN_3$, 100 μL of 5 mg/mL apoGO in 0.1 M buffer, pH 7.4, and 100 μL FAD at various concentrations. This mixture was stirred for 5 min-

utes before adding benzoquinone solution (6.5 mL, 25 mM). Oxidation current response was measured at a constant potential bias of 450 mV vs. AgCl.

An FAD dose-response curve was obtained by plotting change in oxidation current vs. final FAD concentration, shown in Figure 3. Oxidation current increased in response to increasing concentrations of FAD over the dynamic range, $1.71 \times 10^{-11}$ M to $1.71 \times 10^{-7}$ M FAD.

The detection limit of an immunoassay is inversely proportional to the affinity constant of the antibody, in this case $3 \times 10^{8}$ $M^{-1}$. The electrochemically determined detection limit of $1.71 \times 10^{-11}$ M is therefore well beyond the theoretical detection limit of $3 \times 10^{-9}$ M for this antibody-antigen system.

(B) Reconstitution in Solid Phase:

Apoglucose oxidase was immobilized in a polyacrylamide membrane deposited onto the working electrode surface. Reactivation of immobilized apoGO by FAD diffusing into the membrane from a bulk solution containing necessary reagents was investigated by amperometric measurement at a constant potential bias.

The same two-electrode system described above was used. The electrodes were modified to accommodate the membrane by epoxying a ceramic ring (1 cm inner diameter) to the electrode surface, forming a cell having walls of depth about 1 mm. The electrode surface served as the bottom of the cell. The electrodes were electrochemically cleaned by scanning at a rate of 100 mV/sec from -1.2V to +0.2V vs. AgCl in 0.5 M $H_2SO_4$ for 20 min and rinsing in distilled water.

Apoglucose oxidase was immobilized in polyacrylamide by mixing 20 mg apoGO in 100 µL 0.1 M buffer, pH 7.4, and 100 µL 40% acrylamide in a 10 mL beaker over ice. Ammonium persulfate (10 µL, 10%) and 5 µL TEMED (N,N,N',N'-

tetramethylethylenediamine) were then added and stirred before pipetting 20 μL of this solution into the electrode cell. The final applied membrane contained a total of approximately 200 μg apoGO. After the membrane gelled, it was rinsed with distilled water and immersed in a 20 mL reaction vessel containing 5 mL of a benzoquinone stock solution (25 mM) and 3 mL of a 100 mM glucose/100 mM $NaN_3$ stock solution (final concentrations: benzoquinone - 15.6 mM; glucose/$NaN_3$ - 37.5 mM each). The solution was stirred constantly at a stir speed of 3, and hydroquinone oxidation current was measured at 600 mV vs. AgCl. Aliquots of FAD (100 μL) of various concentrations were added to the reaction mixture and change in oxidation current recorded after 20 minutes or when current stabilized.

Prior to electrochemical experiments, the ability of FAD, benzoquinone and glucose to permeate the membrane was confirmed by immersing the white membrane in each solution and observing a color change (yellow for FAD, brown for benzoquinone/glucose) on cutting a cross section of the soaked membrane.

Oxidation current increased in response to increasing concentrations of FAD (up to 93% for $3 \times 10^{-6}$ M FAD final concentration). An FAD dose-response curve was obtained over a range $3 \times 10^{-10}$ M to $3 \times 10^{-6}$ M FAD final concentration, shown in Figure 4.

## Example 3

(Minimizing Drift)

(A) In the measurements described in the examples above using sputtered Pt as the working electrode, baseline drift was consistently substantial, typically 2 μA/hr with over a 200% increase in current. This drift may be due to adsorption of reagents onto the electrode surface, resulting in fouling of the electrode. In particular, the

benzoquinone/glucose solution was observed to turn viscous and murky in color with time. Benzoquinone, known to be sensitive to both light and to oxygen, can conceivably change over time, possibly reacting with glucose and accumulating on the electrode surface. Measurements made with the carbon disk electrode resulted in less drift, possibly due to the favorable electrochemical and physical properties of carbon or better cleaning with this structure.

To minimize drift from sputtered Pt electrodes, a dynamic method of amperometric measurement (cyclic voltammetry) was adapted in which potential was continuously cycled over a fixed range. Cyclic voltammetry affords continuous electrochemical cleaning and reactivation of the electrode surface. Cyclic voltammetry also allows a visual representation of the spectrum of electrochemical activity over a wide potential range.

(A) Cyclic Voltammetry With a Carbon Disk Electrode:

Cyclic voltammetry was investigated as a measurement technique to overcome problems which result in drift of baseline current. Current response to FAD in the previously described carbon disk/AgCl two-electrode system was measured by cyclic voltammetry. The same BAS CV-27 potentiostat and settings were used. All measurements were made in a 15 mL reaction vessel. The baseline reaction mixture consisted of a 6 mL solution of 500 μM benzoquinone, 5 mM glucose and 5 mM $NaN_3$ in 0.1 M phosphate buffer, pH 7.4. A stock apoGO solution (20 μL, 10 mg/mL) was added to this solution to produce a final concentration of approximately 10 μg/mL apoGO. This reaction mixture was stirred constantly at a setting of 3. Potential was scanned from -800 mV to +800 mV vs. AgCl at a scan rate of 100 mV/sec. Cyclic voltammograms were recorded with an HP700B4 X-Y recorder at 5 min intervals. After the baseline scans stabilized, 100 μL of FAD at

various concentrations was added. The extent of the enzyme reaction was measured by monitoring both (hydroquinone) oxidation and (benzoquinone) reduction current peaks at 500 mV and -270 mV vs. AgCl, respectively, after twenty minutes.

Both oxidation and reduction baseline currents decreased initially, but stabilized after about 10 minutes with subsequent cyclic voltammograms becoming superimposable. Oxidation current increased (up to 200% for 1.6 x $10^{-6}$ M FAD) accompanied by decreased reduction current (up to 90%) upon adding increasing concentrations of FAD. This trend continued until the reduction peak disappeared, at which point a reversal of current direction for both oxidation and reduction peaks occurred. Subsequently, the oxidation current began to decrease and the reduction peak began to increase, indicating a depletion of benzoquinone and a shift of the benzoquinone/hydroquinone equilibrium favoring production of benzoquinone.

Two FAD dose-response curves were obtained by plotting percent change in oxidation and reduction currents vs. FAD final concentration over the range 1.6 x $10^{-10}$ M to 1.6 x $10^{-6}$ M FAD (Figure 5). Percent changes in oxidation current were generally greater than percent changes in reduction current. This is because the initial hydroquinone oxidation current was small compared to the initial benzoquinone reduction current, since hydroquinone is generated as a reaction product from excess benzoquinone. Thus, any change in oxidation current, although smaller in absolute magnitude than a corresponding change in reduction current, is a greater percent of total current.

(B) Comparison Of Carbon, Platinum and Silver Electrode Systems:

Performance of carbon disk vs. AgCl wire and sputtered Pt vs. chloridized sputtered Ag working vs. reference electrode systems was compared. Since apoenzyme reactiva-

tion rate is diffusion-limited, the size of the reaction vessel and the overall reaction volume were minimized to decrease response time.

The sputtered Pt/Ag electrode cell described above was used in the two-electrode configuration. An additional electrode cell similar in size was constructed by inverting the BAS carbon disk electrode so that the carbon disk constituted the cell floor, and cell walls were formed by inserting the tip snugly into a ring cut from Tygon® tubing. A chloridized silver wire coil was used as the reference/counter electrode. The electrode wire was tightly coiled in order to increase the reactive surface area of an electrode small enough to fit into a reaction cell which could be immersed in a volume of 100 μL. The reaction mixture consisted of 100 μL of 0.5 mM benzoquinone and 5 mM each of glucose and $NaN_3$ in 0.1 M phosphate buffer, pH 7.4. Oxidation and reduction currents were measured at +500 mV and -200 mV vs. AgCl, respectively, and monitored by cyclic voltammetry using the same parameters described above. GO (10 μL, 10 mg/mL) was carefully pipetted into both cells, and the change in current recorded.

While baseline current of the cell using the carbon electrode stabilized within 10 min, baseline of the sputtered Pt vs. Ag cell decreased at a rate of 10 μA/hr. This drift was likely due to the quality of sputtered Pt and/or sputtered Ag. Despite this drift, addition of GO resulted in immediate increases in oxidation current (159% for the carbon disk vs. AgCl coiled wire, 10% for sputtered Pt vs. sputtered Ag) and decreases in reduction current (70% for the carbon disk vs. AgCl coiled wire and 53% for sputtered Pt vs. sputtered Ag). Response time was less than 10 seconds. The effect of the drift in baseline for the sputtered Pt vs. sputtered Ag electrode cell was reflected in the substantially smaller overall response to GO relative to

the carbon disk electrode system. As the direction of the drift was negative (i.e., opposite the direction of oxidation current response) it counteracted the oxidation current response, resulting in a smaller increase than that obtained for the carbon disk vs. AgCl wire coil electrode.

(C) Source of Drift:

The same sputtered Pt/Ag electrodes described above were used in two different two-electrode configurations: 1) sputtered Pt vs. sputtered Ag, and 2) sputtered Pt vs. chloridized Ag coil. Baseline current was measured by cyclic voltammetry as described above.

While baseline current of the electrode cell using sputtered Ag as reference continued to decrease over time (1 hr), the baseline current for the cell in which AgCl wire was used as the reference electrode stabilized substantially within 10 minutes. Most likely, the chloridized sputtered Ag did not function satisfactorily as a stable reference electrode, due to poor quality of sputtered Ag and lack of an adhesion layer. Use of a chloridized Ag wire coil as the reference/counter electrode reduced drift relative to the chloridized sputtered Ag.

Although use of a small reaction cell and volume improved response time, responsiveness of the polyacrylamide membrane-covered electrode was suboptimal due to non-uniform membrane thickness and slow diffusion through the membrane. Consequently, another means of immobilizing the enzyme was sought.

## Example 4

(Alternate Enzyme Immobilization)

Drying solutions of enzyme (apoGO or GO) directly onto the electrode surface was investigated as a means of immobilization. The sputtered Pt vs. chloridized Ag wire coil two electrode system described above was used. GO and

apoGO were dried on electrode surfaces by pipetting 10 μL of enzyme solution (100 mg/mL in 0.1 M phosphate buffer, pH 7.4) onto the electrode cell and blowing the surface dry with $N_2$ gas. This procedure was repeated five times before rinsing with distilled water and blowing dry a final time. Next, 100 μL of either 1 mM benzoquinone (for GO) or 0.5 mM benzoquinone and 5 mM glucose (for apoGO) was added to the electrode cell in 0.1 M phosphate buffer, pH 7.4, and current was measured from 0 to +800 mV by cyclic voltammetry as described above. Scanning in only the positive potential range eliminated depletion of benzoquinone due to electrochemical reduction. After baseline current stabilized, either 5 μL of 10 mM glucose or 10 μL of a $10^{-6}$ M FAD stock solution was carefully pipetted into appropriate cells and cyclic voltammograms recorded at 1 min intervals. Oxidation current at 800 mV increased immediately upon addition of respective reagents (46% for glucose, 8% for FAD) with a response time of less than 10 seconds.

## Example 5

(Demonstration of Layered Configuration)

Filter paper was investigated as a dual acting substrate for 1) immobilization of apoGO by absorption into the paper itself (1st layer); and 2) incorporation of a polyacrylamide membrane as a second layer. Permeability of the membrane to FAD and to an FAD-theophylline conjugate ("FAD-T") and reactivation of apoGO absorbed in the filter paper were also investigated by cyclic voltammetry.

The same sputtered Ag/Pt two electrode cell, setup and equipment used for cyclic voltammetric measurements described above were used. Whatman #1 filter paper was used as the substrate for the polyacrylamide membrane. Acrylamide (200 μL, 20% solution in 0.1 M phosphate buffer, pH 7.4) was polymerized by adding 10 μL of 10% ammonium per-

sulfate and 5 μL of TEMED, stirring quickly over ice and pouring onto the filter paper. The filter paper was laid over the mouth of a 50 mL beaker to minimize seepage of the membrane solution through the filter paper. After the filter paper became saturated, a thin layer of acrylamide solution about 1 cm x 0.5 cm in surface area remained on top. The membrane was left until polymerization was complete. After the filter paper was dry, circles were punched out with a #5 (1 cm diameter) cork borer.

ApoGO (50 μg) was immobilized in the filter paper by pipetting 5 μL of an apoGO solution (10 mg/mL apoGO in 0.1 M phosphate buffer) onto the reverse side of the filter paper (the side without polyacrylamide) and letting it absorb into the paper.

For electrochemical measurements, a benzoquinone solution (5 μL of 500 μM benzoquinone, 5 mM glucose and 5 mM $NaN_3$) was added to the electrode to wet the surface. The membrane/filter paper circle with absorbed apoGO was inserted into the cell, apoGO side down, to fit snugly against the electrode surface. Oxidation current was measured by scanning from 0 to +800 mV vs. AgCl at a scan rate of 100 mV/sec. After the baseline current stabilized, 5 μL of 0.1 M buffer solution was carefully pipetted onto the membrane surface as a control and current measured for at least 30 min. Next, 5 μL aliquots of either FAD ($10^{-8}$ - $10^{-6}$ M) or FAD-T ($10^{-6}$ M) were pipetted onto the membrane surface and current responses recorded.

Baseline current stabilized to less than 1% per minute drift within 10 minutes. No current change was recorded on addition of 0.1 M phosphate buffer In contrast, dose-dependent increases in current over baseline were observed with increasing concentrations of FAD: from 240 nA at $10^{-8}$ M FAD to 3 μA at $10^{-6}$ M FAD with response

times of less than 30 seconds. Similarly, a 2 μA current increase occurred with addition of $10^{-6}$ M FAD-T.

Thus, filter paper was shown to be an effective substrate for absorbing apoGO and supporting a polyacrylamide membrane in a two-layer configuration. FAD ($10^{-8}$ M to $10^{-6}$ M) and FAD-T conjugate ($10^{-6}$ M) were able to permeate through the membrane to reactivate apoGO, resulting in an increase in oxidation current at the electrode.

## Example 6

(Alternate Configuration)

A hapten-displacement device of the invention was prepared, using a hydrogel-immobilized layer of anti-theophylline antibody:FAD-T complexes (Ab:FAD-T). The Ab:FAD-T complex was immobilized in polyacrylamide using filter paper as a substrate, as described above. The dose-response relationship for unlabeled theophylline was measured by cyclic voltammetry.

Anti-theophylline monoclonal IgG antibody (affinity constant 3 x $10^{-8}$ L/mol) and theophylline were obtained from OEM Concepts, Inc., and FAD-T conjugate was obtained from Miles Diagnostics group. Methods were the same as described above, except for inclusion of 50 μL $10^{-8}$ M anti-theophylline monoclonal antibody (0.3 μg) and 50 μL $10^{-8}$ M FAD-T conjugate in the 200 μL of 20% acrylamide. This membrane solution was incubated for 30 min in a 37°C water bath to allow antibody:FAD-T complex formation.

Prior to measuring electrochemical responses, 5 μL of 0.1 M phosphate buffer and 5 μL of (500 μM benzoquinone + 5 mM glucose) were applied to the membrane as separate controls. Similarly, 5 μL aliquots of 10 μM and 100 μM theophylline were added to determine theophylline dose response.

Baseline current ranged from 0.5 to 1.5 μA. Current increase was minimal on addition of controls (0% for

0.1 M phosphate buffer and 5% for the benzoquinone/glucose control). The small current response to the benzoquinone/glucose control is attributable to excess unbound FAD-T conjugate in the membrane. In contrast, dose-dependent current increases were observed with theophylline addition: 19% for 10 μM theophylline and up to 650% for 100 μM theophylline with a response time of less than 30 seconds.

WHAT IS CLAIMED:

1. A device for detecting an analyte within a liquid sample, which device comprises:

a surface comprising a working electrode;

a reference electrode;

a first member of a reversibly inactivated enzyme-reactivating agent set, wherein said reversibly inactivated enzyme and said reactivating agent together form an active enzyme when reconstituted, said active enzyme being capable of catalyzing a current-generating chemical reaction, said first member being immobilized at said surface;

a first swellable matrix positioned on said first member;

a first specific binding partner, specific for said analyte;

a second specific binding partner capable of competing with said analyte for binding with said first specific binding partner, where either said second specific binding partner or said first specific binding partner is labeled with a second member of said reversibly inactivated enzyme-reactivating agent set, and wherein said second set member bound to said specific binding partner is capable of combining with said first set member and reconstituting said enzyme when said first specific binding partner is not bound to said second specific binding partner, but is incapable of reconstituting said enzyme when said first specific binding partner is bound to said second specific binding partner;

a substrate for said enzyme; and

signal-detecting means attached to said electrodes, capable of detecting a signal generated by said current-generating chemical reaction upon catalysis by said enzyme after reconstitution.

2. The device of claim 1, which further comprises:

a second matrix positioned on said first matrix, said second matrix preventing binding between said first and second specific binding partners in the absence of said sample.

3. The device of claim 1, wherein said reversibly inactivated enzyme-reactivating agent set is selected from the group consisting of:

apoglucose oxidase and FAD;
apoglutathione reductase and FAD;
apocytochrome reductase and FMN;
apo-NADPH:enzyme and FMN;
apolipoamide dehydrogenase and FAD;
apopyridoxine phosphate oxidase and FMN;
apo-horseradish peroxidase and heme; and
apocytochrome C and heme.

4. The device of claim 3, wherein said reversibly inactivated enzyme-reactivating agent set is apoglucose oxidase and FAD.

5. The device of claim 1, wherein said first specific binding partner comprises an antibody specific for said analyte.

6. The device of claim 5 wherein said antibody is entrapped in said first matrix, such that said antibody is prevented from diffusing to said first member.

7. The device of claim 1, wherein said signal is detected by measuring current, voltage, conductivity, or capacitance.

8. The device of claim 1, wherein said analyte comprises theophylline.

9. The device of claim 1, wherein said first and second specific binding partners comprise complementary polynucleotides.

10. The device of claim 1, which further comprises a mediator, said mediator having an oxidation/reduction potential lower than that of said enzyme.

11. The device of claim 10, wherein said mediator is selected from the group consisting of ferrocene, hexacyanoferrate, ferricinium salts, benzoquinone, methylene blue, methyl viologen, benzyl viologen, and poly-viologen.

12. A device for detecting an analyte within a liquid sample, which device comprises:

a surface comprising a working electrode;

a reference electrode;

a first member of a reversibly inactivated enzyme-reactivating agent set, wherein said reversibly inactivated enzyme and said reactivating agent together form an active enzyme when reconstituted, said active enzyme being capable of catalyzing a current-generating chemical reaction, said first member being immobilized at said surface;

a first swellable matrix positioned on said first member;

a first specific binding partner, specific for said analyte;

a second specific binding partner specific for said analyte, where either said first specific binding part-

ner or said second specific binding partner is labeled with a second member of said reversibly inactivated enzyme-reactivating agent set, and wherein said second set member bound to said specific binding partner is capable of combining with said first set member and reconstituting said enzyme when said analyte is not bound to said first and second specific binding partners, but is incapable of reconstituting said enzyme when said analyte is bound to said labeled specific binding partners;

a substrate for said enzyme; and

signal-detecting means attached to said electrodes, capable of detecting a signal generated by said current-generating chemical reaction upon catalysis by said enzyme after reconstitution.

13. The device of claim 12, wherein said reversibly inactivated enzyme-reactivating agent set is selected from the group consisting of:

apoglucose oxidase and FAD;

apoglutathione reductase and FAD;

apocytochrome reductase and FMN;

apo-NADPH:enzyme and FMN;

apolipoamide dehydrogenase and FAD;

apopyridoxine phosphate oxidase and FMN;

apo-horseradish peroxidase and heme; and

apocytochrome C and heme.

14. The device of claim 13, wherein said reversibly inactivated enzyme-reactivating agent set is apoglucose oxidase and FAD.

15. The device of claim 12, wherein said first and second specific binding partners comprise antibodies specific for said analyte.

16. The device of claim 12, further comprising a second swellable matrix positioned on said first swellable matrix, said second matrix comprising said second specific binding partner, said second matrix being permeable to said second specific binding partner and said analyte, said second specific binding partner comprising one member of said reversibly inactivated enzyme/reactivating agent set, said first matrix comprising said first specific binding partner.

17. The device of claim 12, wherein said signal is detected by measuring current, voltage, conductivity, or capacitance.

18. The device of claim 12, wherein said analyte comprises theophylline.

19. The device of claim 12, wherein said analyte comprises a polynucleotide, and said first and second specific binding partners comprise polynucleotides complementary to nonoverlapping portions of said analyte polynucleotide.

20. The device of claim 12, which further comprises a mediator, said mediator having an oxidation/reduction potential lower than that of said enzyme.

21. The device of claim 20, wherein said mediator is selected from the group consisting of ferrocene, hexacyanoferrate, ferricinium salts, benzoquinone, methylene blue, methyl viologen, benzyl viologen, and poly-viologen.

22. A method for determining the presence of a selected analyte within a liquid sample, which method comprises:

providing a device comprising a surface comprising a working electrode; a reference electrode; a first member of a reversibly inactivated enzyme-reactivating agent set, wherein said reversibly inactivated enzyme and said reactivating agent together form an active enzyme when reconstituted, said active enzyme being capable of catalyzing a current-generating chemical reaction, said first member being immobilized at said surface; a first swellable matrix positioned on said first member, a first specific binding partner specific for said analyte; a second specific binding partner capable of competing with said analyte for binding with said first specific binding partner, where either said second binding partner or said first specific binding partner is labeled with a second member of said reversibly inactivated enzyme-reactivating agent set, and wherein said second set member bound to said specific binding partner is capable of combining with said first set member and reconstituting said enzyme when said first specific binding partner is not bound to said second specific binding partner, but is incapable of reconstituting said enzyme when said first specific binding partner is bound to said second specific binding partner; a substrate for said enzyme; and signal-detecting means attached to said electrodes, capable of detecting a signal generated by said current-generating chemical reaction upon catalysis by said enzyme after reconstitution;

applying said sample to said swellable matrix; and

detecting the resulting signal.

23. A method for determining the presence of a selected analyte within a liquid sample, which method comprises:

providing a device comprising a surface comprising a working electrode; a reference electrode; a first member of a reversibly inactivated enzyme-reactivating agent set, wherein said reversibly inactivated enzyme and said reactivating agent together form an active enzyme when reconstituted, said active enzyme being capable of catalyzing a current-generating chemical reaction, said first member being immobilized at said surface; a first swellable matrix positioned on said first member, a first specific binding partner specific for said analyte, a second specific binding partner specific for said analyte, where either said first binding partner or said second specific binding partner is labeled with a second member of said reversibly inactivated enzyme-reactivating agent set, and wherein said second set member bound to said labeled specific binding partner is capable of combining with said first set member and reconstituting said enzyme when said analyte is not bound to said first and second specific binding partners, but is incapable of reconstituting said enzyme when said analyte is bound to said labeled specific binding partners; a substrate for said enzyme; and signal-detecting means attached to said electrodes, capable of detecting a signal generated by said current-generating chemical reaction upon catalysis by said enzyme after reconstitution;

applying said sample to said swellable matrix; and

detecting the resulting signal.

24. A device for detecting a plurality of selected analytes within a liquid sample, which device comprises:

a plurality of detection modules, each module comprising:

a surface comprising a working electrode;

a reference electrode;

a first member of a reversibly inactivated enzyme-reactivating agent set, wherein said reversibly inactivated enzyme and said reactivating agent together form an active enzyme when reconstituted, said active enzyme being capable of catalyzing a current-generating chemical reaction, said first member being immobilized at said surface;

a first swellable matrix positioned on said first member;

a first specific binding partner, specific for said analyte;

a second specific binding partner capable of competing with said analyte for binding with said first specific binding partner, where either said second specific binding partner or said first specific binding partner is labeled with a second member of said reversibly inactivated enzyme-reactivating agent set, and wherein said second set member bound to said specific binding partner is capable of combining with said first set member and reconstituting said enzyme when said first specific binding partner is not bound to said second specific binding partner, but is incapable of reconstituting said enzyme when said first specific binding partner is bound to said second specific binding partner;

a substrate for said enzyme; and

signal-detecting means attached to said electrodes, capable of detecting a signal generated by said current-generating chemical reaction upon catalysis by said enzyme after reconstitution.

25. The device of claim 24, wherein said device further comprises a sample-receiving means which distributes said liquid sample to each detection module.

26. A device for detecting a plurality of selected analytes within a liquid sample, which device comprises:

a plurality of detection modules, each module comprising:

a surface comprising a working electrode;

a reference electrode;

a first member of a reversibly inactivated enzyme-reactivating agent set, wherein said reversibly inactivated enzyme and said reactivating agent together form an active enzyme when reconstituted, said active enzyme being capable of catalyzing a current-generating chemical reaction, said first member being immobilized at said surface;

a first swellable matrix positioned on said first member;

a first specific binding partner, specific for said analyte;

a second specific binding partner specific for said analyte, where either said first specific binding partner or said second specific binding partner is labeled with a second member of said reversibly inactivated enzyme-reactivating agent set, and wherein said second set member bound to said specific binding partner is capable of combining with said first set member and reconstituting said enzyme when said analyte is not bound to said first and second specific binding partners, but is incapable of reconstituting said enzyme when said analyte is bound to said labeled specific binding partners;

a substrate for said enzyme; and

signal-detecting means attached to said electrodes, capable of detecting a signal generated by said current-generating chemical reaction upon catalysis by said enzyme after reconstitution.

27. The device of claim 26, wherein said device further comprises a sample-receiving means which distributes said liquid sample to each detection module.

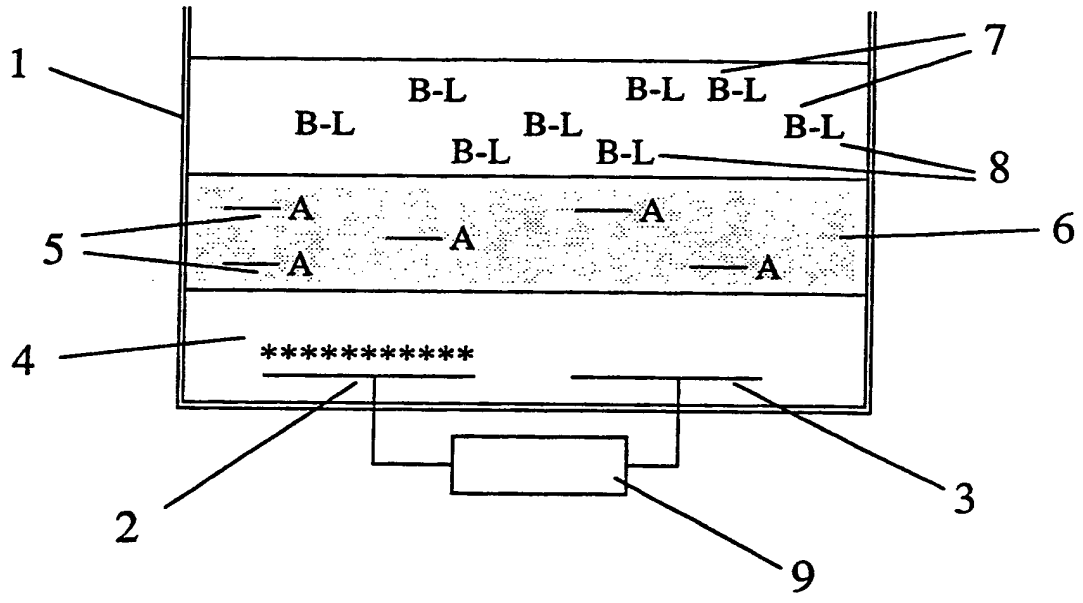



Figure 1

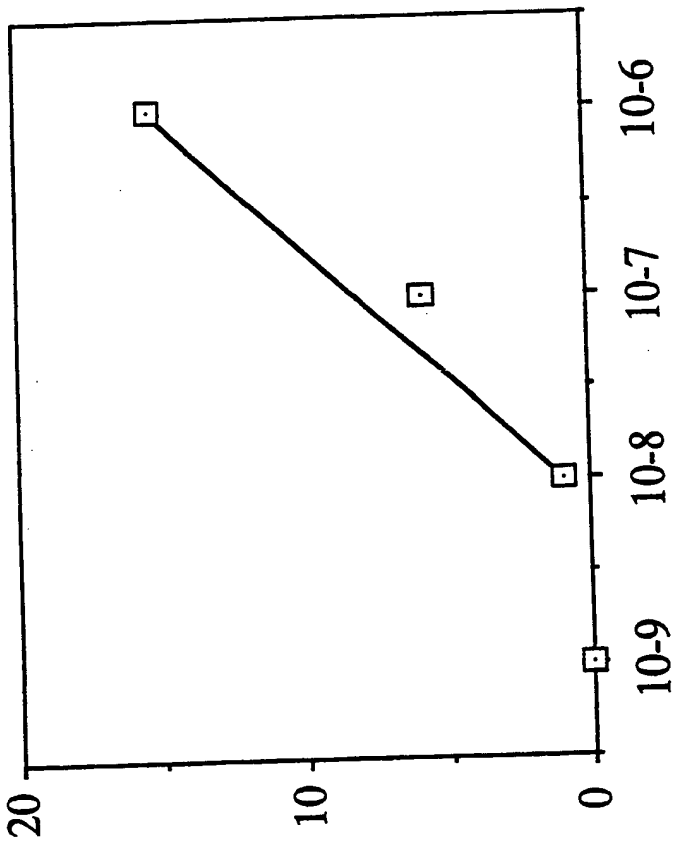



Figure 2

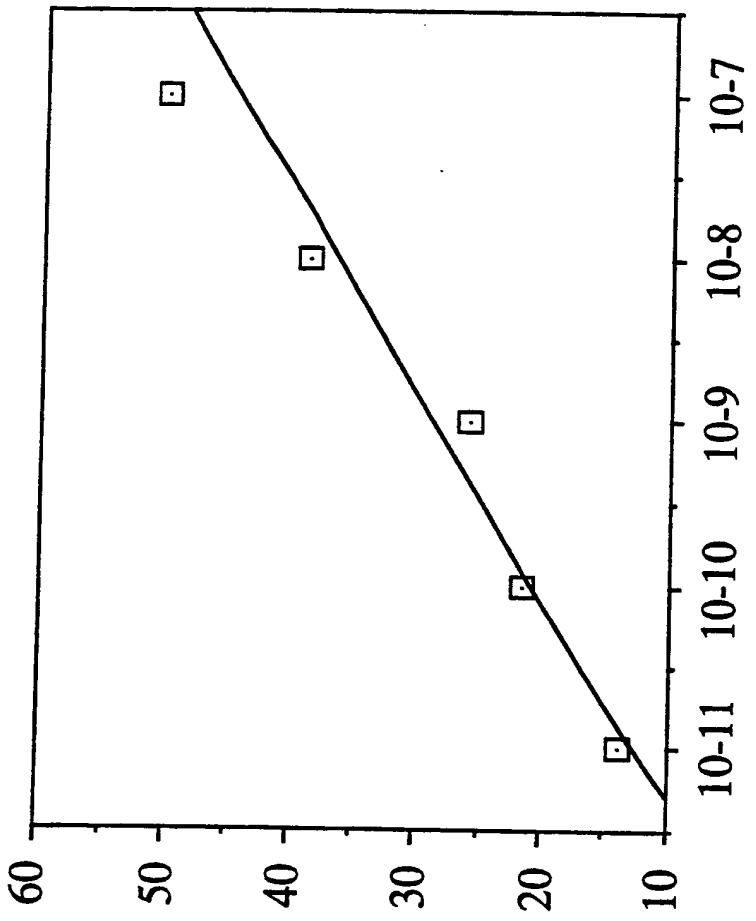



Figure 3

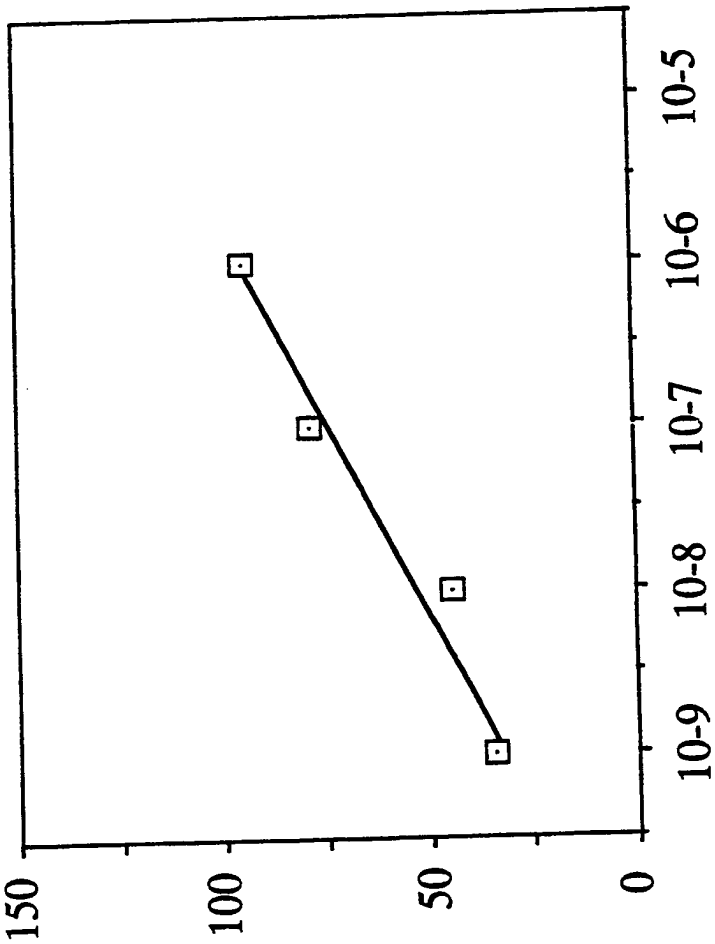



Figure 4

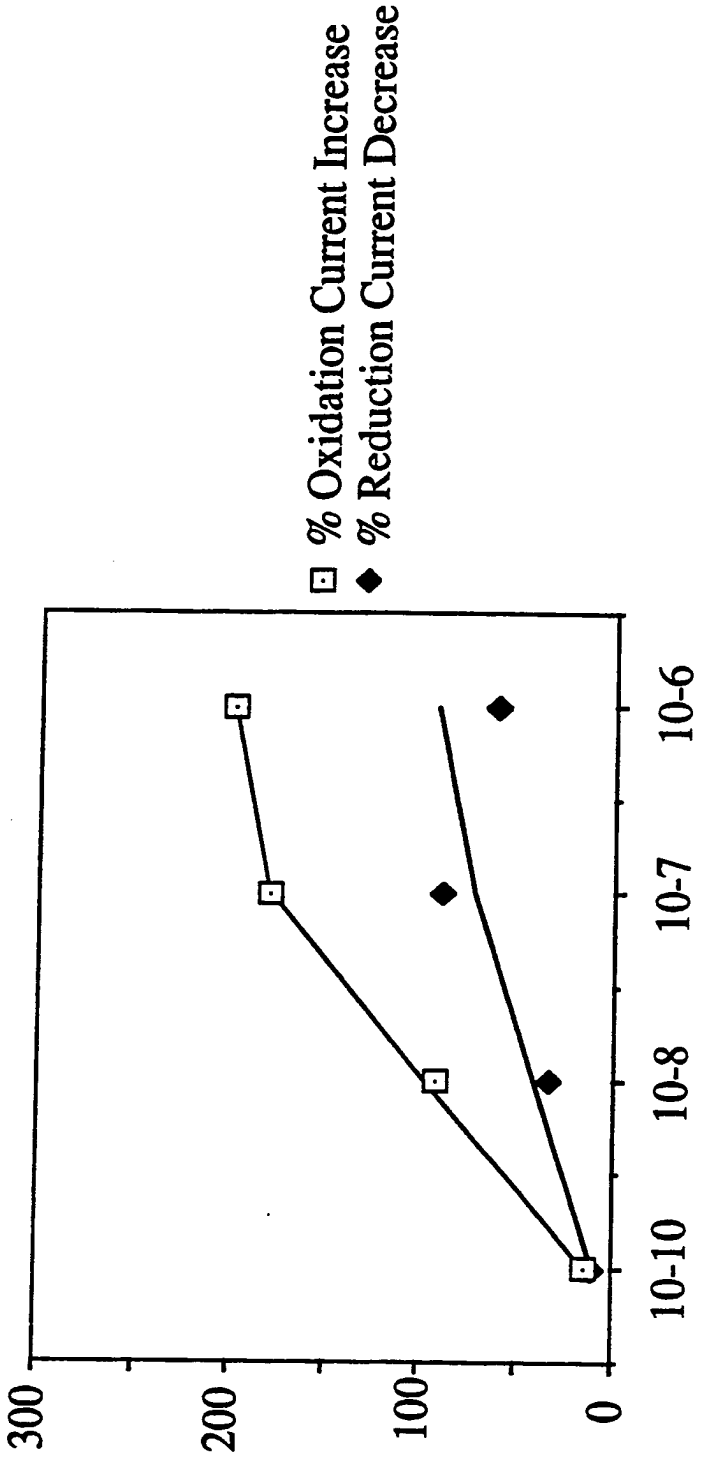



Figure 5

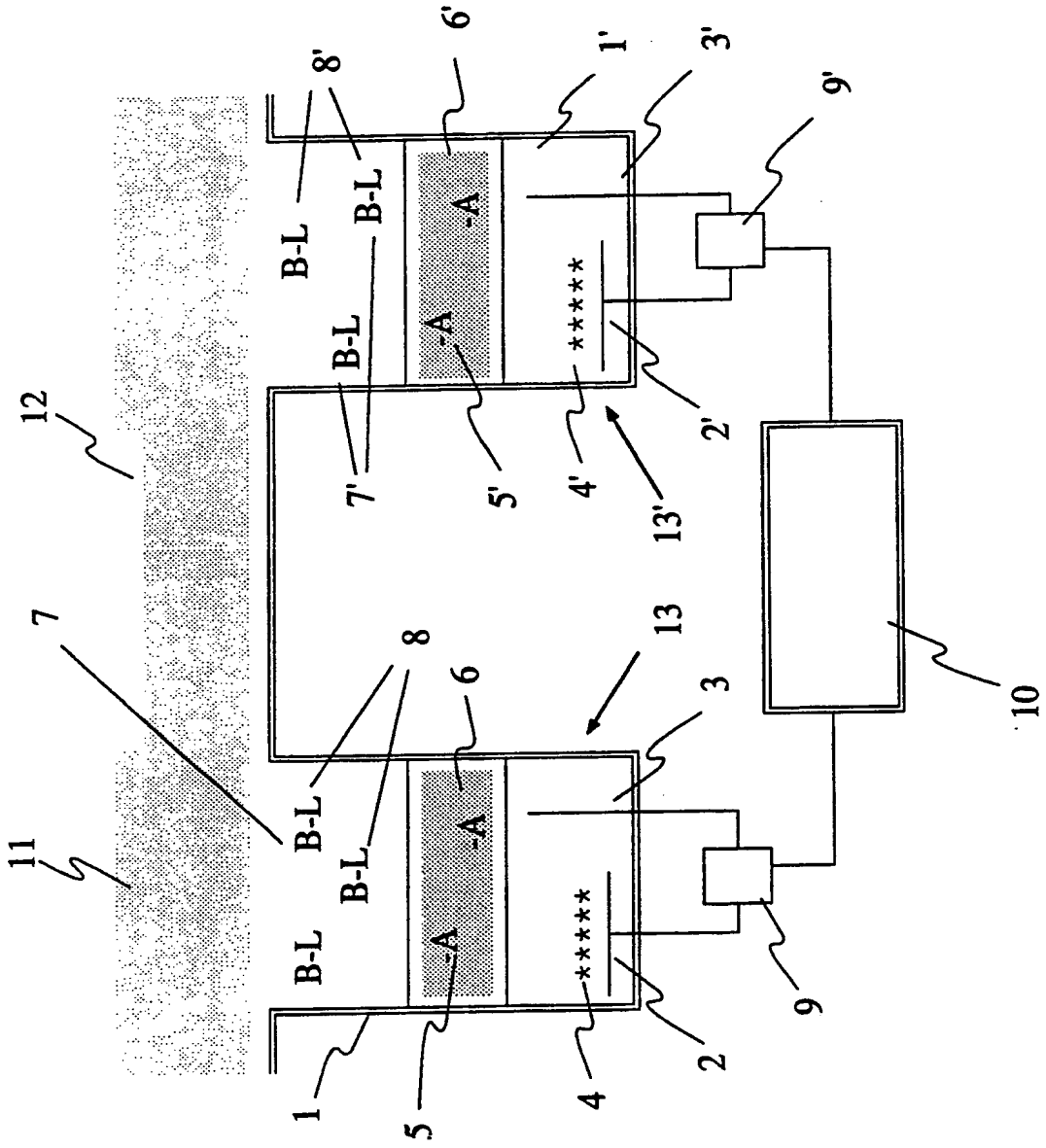



Figure 6

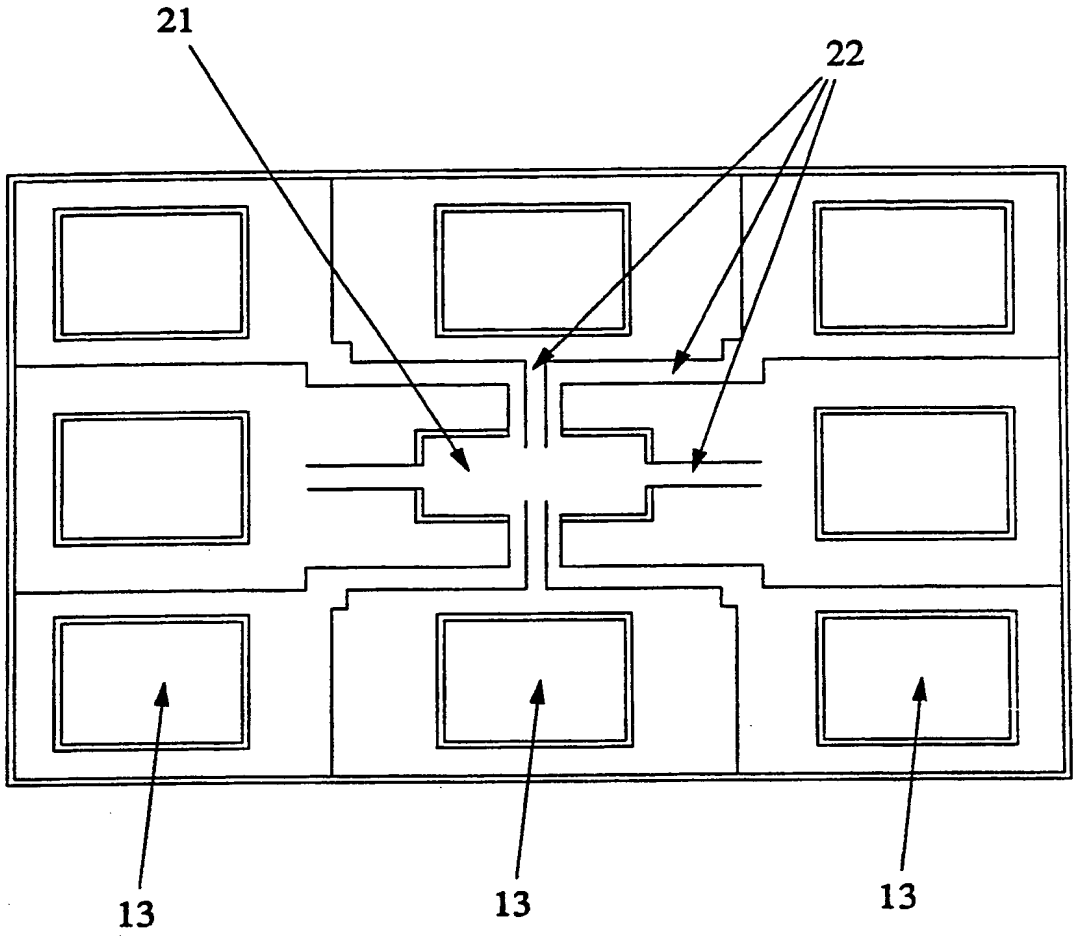



Figure 7

# INTERNATIONAL SEARCH REPORT

International Application No. PCT/US91/02484

## I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) [6]

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC(5): G01N 33/531; C12Q 1/68
U.S. CL.: 435/7.6, 7.7, 7.71

## II. FIELDS SEARCHED

Minimum Documentation Searched [7]

| Classification System | Classification Symbols |
|---|---|
| U.S. | 435/7.6, 7.7, 7.71, 6, 817; 436/806 |

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched [8]

**NONE**

## III. DOCUMENTS CONSIDERED TO BE RELEVANT [9]

| Category * | Citation of Document, [11] with indication, where appropriate, of the relevant passages [12] | Relevant to Claim No. [13] |
|---|---|---|
| Y | US, A, 4,447,526 **(RUPCHOCK ET AL)** 08 May 1984. See column 5, lines 53-68; column 6, lines 1-18; column 7, lines 1-21; column 8, lines 2-32 and column 9, lines 3-5. | 1-11, 22 |
| Y | US, A, 4,224,125 **(NAKAMURA ET AL.)** 23 September 1980. See column 13, lines 12-17 and column 1, lines 30-62. | 1-11, 22 |
| Y | EP, A, 0,142,301 **(FORREST ET AL.)** 22 May 1985. See page 1, lines 19-30; pages 3-5; page 7 and page 8, Table 1. | 1-11, 22 |

* Special categories of cited documents: [10]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

## IV. CERTIFICATION

| Date of the Actual Completion of the International Search | Date of Mailing of this International Search Report |
|---|---|
| 18 June 1991 | 07 AUG 1991 |
| International Searching Authority: ISA/US | Signature of Authorized Officer: Susan C. Wolski (vsh) |

Form PCT/ISA/210 (second sheet) (rev. 11-87)

FURTHER INFORMAT[redacted]ONTINUED FROM THE SECOND SHEET

V. ☐ OBSERVATIONS WHERE CERTAIN CLAIMS WERE FOUND UNSEARCHABLE [1]

This international search report has not been established in respect of certain claims under Article 17(2) (a) for the following reasons:

1. ☐ Claim numbers , because they relate to subject matter [2] not required to be searched by this Authority, namely:

2. ☐ Claim numbers , because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out [3], specifically:

3. ☐ Claim numbers_____, because they are dependent claims not drafted in accordance with the second and third sentences of PCT Rule 6.4(a).

VI. ☒ OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING [2]

This International Searching Authority found multiple inventions in this international application as follows:

**(See attachment sheet)**

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims of the international application.

2. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims of the international application for which fees were paid, specifically claims:

3. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claim numbers: **1–11, 22**

4 ☐ As all searchable claims could be searched without effort justifying an additional fee, the International Searching Authority did not invite payment of any additional fee

Remark on Protest

☐ The additional search fees were accompanied by applicant's protest.

☐ No protest accompanied the payment of additional search fees.

ATTACHMENT TO PCT/ISA/210
UNITY OF INVENTION IS LACKING

I. Claims 1-11, 22 Drawn to a first device and method for detecting an analyte in a liquid sample by competitive assay. Class 435, subclass 7.7.

II. Claims 12-21 Drawn to a second device for detecting an analyte in a liquid sample by sandwich assay. Class 435, subclass 7.7.

III. Claim 23 Drawn to a method for detecting an analyte in a liquid by sandwich assay. Class 435, subclass 7.7.

IV. Claims 24-25 Drawn to a third device for detection of a plurality of analytes in a liquid sample by competitive assay. Class 435, subclass 7.7.

V. Claims 26-27 Drawn to a fourth device for detection of a plurality of analytes in a liquid sample by sandwich assay. Class 435, subclass 7.7.